REMARKS

Claims 1, 2, 4, 6, 7, 10-16, 18, 20-24 and 43-49 are pending. No new matter has been added by way of the present amendment. For instance, the paragraph at pages 46-47 has been amended to correct a minor typographical error. Also, Applicants have amended the specification to include the textual subject matter recited in claims 48 and 49. This subject matter was incorporated by reference into the specification by way of the reference made to the particular hybridization method of Sambrook et al. In particular, at page 46, lines 8-15 of the present specification it is stated:

> A DNA fragment comprising the nucleotide sequence of SEQ. ID. No.: 4 or parts of it can be used as a probe for isolating PPO genes from *Chlamydomonas* or plant DNA libraries according to the hybridization method described by Sambrook et al., Molecular Cloning, 2nd edition, pp. 1.90 - 1.110, c. 1989 by Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

A copy of the incorporated subject matter is attached hereto. Applicants' representative confirms that the amendatory material added to the specification of the present application (U.S. Application Serial No. 09/331,723, as appearing in the present amendment, consists of the same material incorporated by reference in the referencing application. Specifically, the text added to U.S. Application Serial No. 09/331,723 corresponds to specific text taken from Sambrook et al.,

Molecular Cloning, 2nd edition, pp. 1.90-1.110, c. 1989 by Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (hereinafter Sambrook) in the following manner:

Location of Text Added to 09/331,723	**Location of Text Taken From Sambrook**
After the recitation at page 47, line 4 of "by autoradiography." the sentence "For higher stringencies, the filters may be washed for 60 minutes in 300-500 ml of a solution of 0.2X SSC and 0.1% SDS at 68°C." was added.	Page 1.103, lines 17-20 from the bottom.

This amendment satisfies the requirements set forth in In re Hawkins, 179 U.S.P.Q. 157 (CCPA 1973). Therefore, no new matter has been added. Further, entry of the amendment on the record is respectfully requested. Favorable action on the merits is respectfully requested.

If the Examiner has any questions or comments, please contact Craig A. McRobbie, Reg. No. 42, 874 at the offices of Birch, Stewart, Kolasch & Birch, LLP at the number listed below.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any

additional fees required under 37 C.F.R. §§1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

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Attachment: Sambrook et al., Molecular Cloning, 2nd edition, pp. 1.90-1.110, 1989.

Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

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SCREENING BY HYBRIDIZATION

In 1975, Grunstein and Hogness described a method for in situ lysis of bacterial colonies on nitrocellulose filters and noncovalent attachment of the released DNA to the filters. The DNA could then be hybridized to appropriate radiolabeled nucleic acid probes. This procedure was an extension of the work of Nygaard and Hall (1963), who had shown that DNA could be immobilized on nitrocellulose, and Denhardt (1966) and Gillespie and Spiegelman (1965), who were the first to demonstrate that such fixed nucleic acids could be detected by hybridization. Although minor modifications have been introduced over the years, the protocol originally described by Grunstein and Hogness has proved to be remarkably durable. It remains the most commonly used technique to identify individual bacterial colonies carrying cosmids or plasmids that contain DNA sequences of interest. Methods are presented below to screen bacterial colonies with radiolabeled probes that are longer than 100 nucleotides in length. The preparation of these ^{32}P-labeled probes is described in Chapter 10. Methods for screening bacterial colonies with shorter radiolabeled oligonucleotides are given in Chapter 11. It is easily possible to screen many hundreds of thousands of colonies simultaneously and to recognize colonies that carry recombinant plasmids containing the target sequences. The structure of these plasmids is then verified by restriction analysis and Southern hybridization of minipreparations of plasmid DNA.

Three types of solid supports may be used for in situ hybridization of lysed bacterial colonies—nitrocellulose filters, nylon membranes, and Whatman 541 filter papers. *Nylon membranes* are the most durable of the three and will withstand several rounds of hybridization and washing at elevated temperatures. They are therefore preferred when colonies are to be screened sequentially with a number of different probes. Because different brands of nylon membranes require different treatments, we recommend following the manufacturer's instructions precisely during immobilization and hybridization of DNA. *Whatman 541* filter paper, which has a high wet strength, was first used by Gergen et al. (1979) to screen bacterial colonies. Whatman 541 paper has been used chiefly to screen libraries that have been arranged and stored as cultures of individual colonies in separate wells of microtiter plates (Linbro Scientific). These ordered libraries are duplicated on the surface of agar medium (usually in square petri dishes), and the resulting colonies are then transferred to Whatman 541 paper and lysed either by alkali or by a combination of alkali and heat (Maas 1983). Conditions for hybridization of the immobilized DNA are essentially identical to those established for nitrocellulose filters. Whatman 541 paper has some advantages over nitrocellulose filters: It is cheaper, more durable during hybridization, and less prone to distortion and cracking during drying. However, unless care is taken during the denaturation step (Maas 1983), the strength of the hybridization signal is significantly lower than that obtained from nitrocellulose filters. For routine screening of bacterial colonies, therefore, *nitrocellulose filters* remain the preferred choice as solid supports.

There are many methods available to hybridize radioactive probes in solution to nucleic acids immobilized on filters. These methods differ in the following respects:

- Solvent and temperature used (e.g., 68°C in aqueous solution or 42°C in 50% formamide)
- Volume of solvent and length of hybridization (large volumes for periods as long as 3 days or minimal volumes for times as short as 4 hours)
- Degree and method of agitation (continuous shaking or stationary)
- Use of agents such as Denhardt's reagent or BLOTTO to block the non-specific attachment of the probe to the surface of the solid matrix
- Concentration of the labeled probe and its specific activity
- Use of compounds, such as dextran sulfate (Wahl et al. 1979) or polyethylene glycol (Renz and Kurz 1984; Amasino 1986), that increase the rate of reassociation of nucleic acids
- Stringency of washing following the hybridization

These and other factors affecting hybridization of immobilized nucleic acids are discussed in detail in Chapter 9, pages 9.47–9.51.

Transferring Small Numbers of Colonies to Nitrocellulose Filters

This procedure is used when it is necessary to screen a small number of bacterial colonies (100–200) that are dispersed over several agar plates. The colonies are consolidated on a master agar plate and on a nitrocellulose filter laid on the surface of a second agar plate. After a period of growth, the colonies are lysed in situ. The master plate is stored at 4°C until the results of the screening procedure become available.

1. Place a nitrocellulose filter (Millipore HAWP or equivalent) on an agar plate containing the selective antibiotic.

   It is not necessary to use a detergent-free or sterilized filter in this procedure; such filters are required only when small numbers of bacteria are used as inocula, for example, when the transformation mixture is plated directly on the surface of the filter. However, the filter should be handled with gloved hands, since finger oils prevent wetting of the filter and affect DNA transfer.

2. Using sterile toothpicks, transfer individual bacterial colonies onto the filter and then onto a master agar plate that contains the selective antibiotic but no filter. Make small streaks 2–3 mm in length (or dots) arranged in a grid pattern. Each colony should be streaked in an identical position on both plates. Up to 100 colonies can be streaked onto a single 90-mm plate. Finally, streak a colony containing a nonrecombinant plasmid (e.g., pBR322) onto both the filter and the master plate. This negative control is often useful and sometimes necessary to discriminate between specific annealing of the radioactive probe to a recombinant plasmid and nonspecific background hybridization.

3. Invert the plates and incubate them at 37°C until the bacterial streaks have grown to a width of 0.5–1.0 mm.

   At this stage, when the bacteria are still growing rapidly, the filter may be transferred to an agar plate containing chloramphenicol (170–200 μg/ml) and incubated for 12 hours at 37°C (Hanahan and Meselson 1980, 1983). This amplification step is necessary only when the copy number of the recombinant plasmid is expected to be low (e.g., if an unusually large segment [> 10 kb] of foreign DNA has been inserted) or when highly degenerate oligonucleotides are to be used as probes. Under normal circumstances, cloned DNA sequences can be detected very easily by hybridization without prior amplification of the recombinant plasmid. Amplification can be carried out only with vectors that replicate in a relaxed fashion (see page 1.3).

4. Mark the nitrocellulose filter in three or more asymmetric locations by stabbing through it and into the agar beneath with an 18-gauge needle attached to a syringe containing waterproof black drawing ink. Mark the master plate in approximately the same locations.

   With practice, it is possible to avoid the use of ink (which can be messy) by punching holes through the filter into the underlying agar with an empty 18-gauge needle. After hybridization, backlighting can be used to align the holes in the filter with the marks in the agar.

5. Seal the master plate with Parafilm and store it at 4°C in an inverted position until the results of the hybridization reaction are available.

6. Lyse the bacteria and bind the liberated DNA to the nitrocellulose filter by one of the two procedures described on pages 1.98 and 1.100.

Replicating Colonies onto Nitrocellulose Filters

Two methods are given here for replicating colonies onto nitrocellulose filters. Method 1 (Hanahan and Meselson 1980, 1983) is used when it is necessary to screen large numbers of colonies by hybridization (e.g., when plating out bacteria transformed by a cDNA library constructed in a plasmid vector). In this case, bacteria are plated directly from a transformation mixture onto detergent-free nitrocellulose filters, and replica filters are prepared by filter-to-filter contact. Method 2 is used to transfer many bacterial colonies simultaneously from the surfaces of agar plates to nitrocellulose filters. This method works with bacterial colonies of any size, but small colonies (0.1–0.2 mm) give the best results; they produce sharper hybridization signals and smear less than larger colonies. As many as 2×10^4 colonies per 138-mm filter or 10^4 colonies per 82-mm filter can be screened using this technique.

METHOD 1

1. Number dry, detergent-free nitrocellulose filters (Millipore HATF or equivalent) with a soft-lead pencil or a ballpoint pen, wet them with water, and sandwich them between dry Whatman 3MM papers. Wrap the stack of filters in aluminum foil, and sterilize them by autoclaving (15 lb/sq. in. on liquid cycle). Prepare enough filters to make a master and two replicas of each plate.

2. Using sterile, blunt-ended forceps (e.g., Millipore forceps), lay a sterile filter, numbered side down, on a day-old LB (or SOB) agar plate containing the appropriate antibiotic. When the filter is thoroughly wet, peel it from the plate and replace it, numbered side up, on the surface of the agar.

3. Apply the bacteria, in a small volume of liquid, to the center of the filter on the surface of the agar plate. A large filter (138-mm diameter) will accommodate up to 0.5 ml of liquid containing 2×10^4 viable bacteria. A small filter (82-mm diameter) will accommodate up to 0.2 ml of liquid containing approximately 10^4 bacteria. Using a sterile glass spreader, disperse the fluid evenly over the surface of the filter. Leave a border 2–3 mm wide at the edge of the filter free of bacteria. After spreading the inoculum, incubate the plate (noninverted) with the lid ajar for a few minutes in a laminar flow hood to allow the inoculum to evaporate. Then close the lid, invert the plate, and incubate at 37°C until small colonies (0.1–0.2-mm diameter) appear (about 8–10 hours).

4. If desired, replica filters may be prepared at this stage (see step 6). Otherwise, the filter (colony side up) should be transferred to an LB (or SOB) agar plate containing the appropriate antibiotic and 25% glycerol (Hanahan and Meselson 1983). Incubate the plate for 2 hours at 37°C.

5. Seal the plate well with Parafilm, and store it in an inverted position in a sealed plastic bag at −20°C. Replicas can be made after thawing the master plate at room temperature (still in the inverted position).

6. Replica filters are made as follows:

   a. Prepare in advance a stack of Whatman 3MM papers (one for each filter plus a few spares) cut to a size slightly larger than the filters. Sterilize the stack of 3MM papers by autoclaving for 10 minutes at 10 lb/sq. in. on liquid cycle.

   b. Peel the master nitrocellulose filter off the storage plate and lay it, colony side up, on a dampened pad of sterile 3MM paper.

   c. Number a damp, sterile nitrocellulose filter and lay it on the master nitrocellulose filter. Take care to prevent air bubbles from becoming trapped between the two filters. This is best done by bending the second filter slightly so that contact is first made between the centers of the filters. Be careful not to move the filters relative to one another once contact has been made. Try to arrange the filters so that they do not overlap exactly; this makes it easier to separate the two filters.

   d. Press the two filters firmly together with a velvet replica-plating tool (Figure 1.14) or with a heavy glass plate (with a 3MM paper between the glass and the filters).

   e. Orient the two filters by making a series of holes in the pair of filters with an 18-gauge needle.

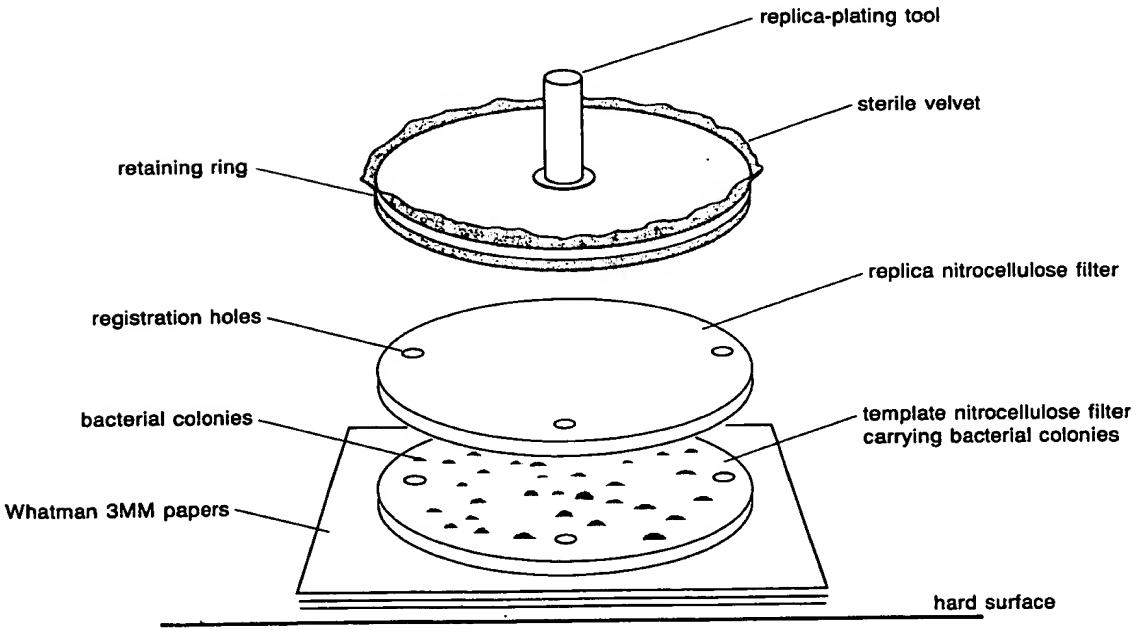

FIGURE 1.14
Use of a replica-plating tool to replicate bacterial colonies growing on nitrocellulose filters.

f. Peel the filters apart. Lay the replica on a fresh LB (or SOB) agar plate containing the appropriate antibiotic and incubate the plate at 37°C until colonies appear (4–6 hours).

At this stage, when the bacteria are still growing rapidly, the filter may be transferred to an agar plate containing chloramphenicol (170–200 μg/ml) and incubated for 12 hours at 37°C (Hanahan and Meselson 1980, 1983). This amplification step is necessary only when the copy number of the recombinant plasmid is expected to be low (e.g., if an unusually large segment [> 10 kb] of foreign DNA has been inserted) or when highly degenerate oligonucleotides are to be used as probes. Under normal circumstances, cloned DNA sequences can be detected very easily by hybridization without prior amplification of the recombinant plasmid. Amplification can be carried out only with vectors that replicate in a relaxed fashion (see page 1.3).

g. Replace the master nitrocellulose filter on a fresh LB (or SOB) agar plate containing the appropriate antibiotic and 25% glycerol (Hanahan and Meselson 1983). Incubate the plate for 1 hour at 37°C, and then freeze it as described in step 5.

If necessary, more replicas can be prepared from the master in an identical manner. Key the second and subsequent replicas to the existing holes in the master filter. Return both filters to their respective plates. If the master filter is to be used to make more than two replicas, it should be incubated for a few hours to allow the colonies to regenerate. Generally, making two to three replicas presents no problem. However, should more replicas be required, it is essential that the bacterial colonies be extremely small (~0.1-mm diameter) at the time of replication in order to avoid smearing. To avoid contamination, filters containing copies of valuable libraries are best handled in a laminar flow hood.

7. Lyse the bacteria and bind the liberated DNA to the replica filters using one of the two methods described on pages 1.98 and 1.100.

METHOD 2

1. Plate the bacterial suspension directly on the surfaces of LB (or SOB) agar plates containing the appropriate antibiotic. Plates that are 2–3 days old give the best results because they absorb the inoculum more readily. Leave the plates, with their lids ajar, in a laminar flow hood until the surface of the agar is quite dry. Close the lids and incubate the plates in an inverted position for 12–14 hours at 37°C. Chill the plates for 30–60 minutes at 4°C.

2. Number a dry, detergent-free nitrocellulose filter (Millipore HATF or equivalent) with a soft-lead pencil or a ballpoint pen, and sterilize it as described in step 1, page 1.93. Place the filter, numbered side down, on the surface of the LB (or SOB) agar medium, in contact with the bacterial colonies, until it is completely wet. Mark the filter and the underlying medium in three or more asymmetric locations by stabbing through the filter with an 18-gauge needle attached to a syringe containing waterproof black drawing ink.

   Although sterile filters are preferred, nonsterile filters can also be used as long as no more replicas are to be made from the master plate.

   With practice, it is possible to avoid the use of ink (which can be messy) by punching holes through the filter into the underlying agar with an empty 18-gauge needle. After hybridization, backlighting can be used to align the holes in the filter with the marks in the agar.

3. Using blunt-ended forceps (e.g., Millipore forceps), peel off the filter.

4. At this stage, several options are available:

   - The bacteria adhering to the filter may be immediately lysed and the liberated DNA bound to the filter using one of the two methods described on pages 1.98 and 1.100.
   - The filter may be placed, colony side up, on the surface of a fresh LB (or SOB) agar plate containing the appropriate antibiotic. The colonies are lysed after a few hours of incubation, when they have grown to a size of 2–3 mm. This method is necessary only when the transfer of the colonies to the filter is poor or uneven, which is not usually the case.
   - The filter may be transferred to an agar plate containing chloramphenicol (170–200 μg/ml) and incubated for 12 hours at 37°C (Hanahan and Meselson 1980, 1983). This amplification step is necessary only when the copy number of the recombinant plasmid is expected to be low (e.g., if an unusually large segment [>10 kb] of foreign DNA has been inserted) or when highly degenerate oligonucleotides are to be used as probes. Under normal circumstances, cloned DNA sequences can be detected very easily by hybridization without prior amplification of the recombinant plasmid. Amplification can be carried out only with vectors that replicate in a relaxed fashion (see page 1.3).
   - The filter may be used to prepare a second replica. The filter is placed, colony side up, on the surface of a fresh LB (or SOB) agar plate

containing the appropriate antibiotic. A second dry nitrocellulose filter is then laid carefully on top of the first and keyed to it. The filter sandwich is incubated for several hours at 37°C, and the plasmids are amplified, if desired, by further incubation on an agar plate containing chloramphenicol. The filters are kept as a sandwich during the subsequent lysis and neutralization steps but are peeled apart before the final wash (Ish-Horowicz and Burke 1981).

5. Incubate the master plate for 5–7 hours at 37°C until the colonies have regenerated. Seal the plate with Parafilm, and store it at 4°C in an inverted position.

Lysis of Colonies and Binding of DNA to Nitrocellulose Filters

Two methods (based on the original procedure of Grunstein and Hogness [1975]) are described to liberate the DNA from bacterial colonies and to bind it to the nitrocellulose filter in situ. The first method can easily be adapted to accommodate large batches of filters, whereas the second is more economical when dealing with only one or two filters. In both cases, try to avoid getting any of the solutions onto the upper surface of the filter and trapping air bubbles under the filter.

METHOD 1

1. Cut four pieces of Whatman 3MM paper to an appropriate size and shape and fit them neatly onto the bottoms of four glass or plastic trays. Cafeteria trays are ideal for this purpose, since they can accommodate batches of up to 25 filters. Saturate each of the pieces of 3MM paper with one of the following solutions:

   - 10% SDS (optional)
   - Denaturing solution (0.5 N NaOH, 1.5 M NaCl)
   - Neutralizing solution (1.5 M NaCl, 0.5 M Tris·Cl [pH 7.4])
   - 2× SSC

   Pour off any excess liquid.

   If the 3MM paper is too wet, the bacterial colonies swell and diffuse during lysis. The hybridization signals then become blurred and attenuated, and it is very difficult to identify individual colonies that give rise to the signal.

2. Using blunt-ended forceps (e.g., Millipore forceps), peel the nitrocellulose filters from their plates and place them, colony side up, on the SDS-impregnated 3MM paper. This treatment limits the diffusion of the plasmid DNA during denaturation and neutralization, resulting in a sharper hybridization signal.

3. After the first filter has been exposed to the SDS solution for 3 minutes, transfer it to the second sheet of 3MM paper, which has been saturated with denaturing solution. Transfer the remainder of the filters in the same order that they were removed from their agar plates. Expose each filter to the denaturing solution for 5 minutes.

   When transferring filters from one tray to another, use the edge of the first tray as a scraper to remove as much fluid as possible from the underside of the filter. Alternatively, remove excess liquid by transferring the filter briefly to a dry paper towel.

   Try to avoid getting fluid on the side of the filter carrying the bacterial colonies.

4. Transfer the filters to the third sheet of 3MM paper, which has been saturated with neutralizing solution. Leave the filters for 5 minutes.

5. Transfer the filters to the last sheet of 3MM paper, which has been saturated with 2× SSC. Leave the filters for 5 minutes.

6. Lay the filters, colony side up, on a sheet of dry 3MM paper. Allow them to dry at room temperature for at least 30 minutes.

7. Sandwich the filters between two sheets of dry 3MM paper. Fix the DNA to the filters by baking for 1–2 hours at 80°C in a vacuum oven.

   Overbaking can cause the filters to become brittle. Filters that have not been completely neutralized turn yellow or brown during baking and chip very easily. The background of nonspecific hybridization also increases dramatically.

8. Hybridize the DNA immobilized on the filters to a ^{32}P-labeled probe as described on pages 1.101–1.104.

   Any filters not used immediately in hybridization reactions should be wrapped loosely in aluminum foil and stored under vacuum at room temperature.

METHOD 2

1. For each filter, make a puddle (0.75 ml) of 0.5 N NaOH on a piece of Saran Wrap. Place a filter, colony side up, on the puddle, stretching the Saran Wrap so that the filter wets evenly. Leave the filter in place for 2–3 minutes.

2. Blot the underside of the filter on a dry paper towel and repeat step 1 using a fresh piece of Saran Wrap and fresh 0.5 N NaOH.

3. Blot the filter again and transfer it to a puddle of 1 M Tris · Cl (pH 7.4) on a fresh piece of Saran Wrap. After 5 minutes, blot the filter and repeat the step using a fresh piece of Saran Wrap and fresh 1 M Tris · Cl (pH 7.4).

4. Blot the filter and transfer it to a puddle of 1.5 M NaCl, 0.5 M Tris · Cl (pH 7.4) on a fresh piece of Saran Wrap. After 5 minutes, blot the filter and transfer it to a piece of dry 3MM paper. Allow the filter to dry at room temperature for 20–30 minutes.

5. Sandwich the filter between two sheets of dry 3MM paper. Fix the DNA to the filter by baking for 2 hours at 80°C in a vacuum oven.

   Overbaking can cause the filters to become brittle. Filters that have not been completely neutralized turn yellow or brown during baking and chip very easily. The background of nonspecific hybridization also increases dramatically.

6. Hybridize the DNA immobilized on the filter to a ^{32}P-labeled probe as described on pages 1.101–1.104.

   Any filters not used immediately in hybridization reactions should be wrapped loosely in aluminum foil and stored under vacuum at room temperature.

Hybridization to Nitrocellulose Filters Containing Replicas of Bacterial Colonies

The following protocol is designed for 30 circular nitrocellulose filters, 82 mm in diameter. Appropriate adjustments to the volumes should be made when carrying out hybridization reactions with different numbers or sizes of filters.

1. Float the baked filters on the surface of a tray of 2× SSC until they have become thoroughly wetted from beneath. Submerge the filters for 5 minutes.

2. Transfer the filters to a glass crystallizing dish containing at least 200 ml of prewashing solution. Stack the filters on top of one another in the solution. Cover the dish with Saran Wrap and transfer it to a rotating platform in an incubator. In this and all subsequent steps, the filters should be slowly agitated to prevent them from sticking to one another. Incubate the filters for 30 minutes at 50°C.

   Important: Do not allow the filters to dry at any stage during the prewashing, prehybridization, or hybridization steps.

Prewashing solution

5× SSC
0.5% SDS
1 mM EDTA (pH 8.0)

3. Gently scrape the bacterial debris from the surfaces of the filters using Kimwipes soaked in prewashing solution. This reduces background hybridization without affecting the intensity or sharpness of positive signals.

4. Transfer the filters to 150 ml of prehybridization solution in a glass crystallizing dish. Incubate the filters for 1–2 hours at the appropriate temperature (i.e., 68°C when hybridization is to be carried out in aqueous solution; 42°C when hybridization is to be carried out in 50% formamide).

Some workers prefer to incubate filters in heat-sealable plastic bags (Sears Seal-A-Meal or equivalent) (see, e.g., Chapter 9, page 9.53). This method avoids problems with evaporation and, because the sealed bags can be submerged in a water bath, ensures that the temperatures during hybridization and washing are correct. The bags must be opened and resealed when changing buffers. To avoid radioactive contamination of the water bath, the resealed bag containing radioactivity should be sealed inside a second, noncontaminated bag.

Often, small bubbles of air form on the surface of the filter as the temperature of the prehybridization solution increases. It is important that these bubbles be removed by occasionally agitating the fluid in the bag; otherwise, the components of the prehybridization solution will not be able to coat the filter evenly. This problem can be minimized by heating the prehybridization solution to the appropriate temperature before adding it to the bag.

The filters should be completely covered by the prehybridization solution. During prehybridization, sites on the nitrocellulose filter that nonspecifically bind single- or double-stranded DNA become blocked by proteins in the BLOTTO.

When ^{32}P-labeled cDNA or RNA is used as a probe, poly(A) at a concentration of 1 μg/ml should be included in the prehybridization and hybridization solutions to prevent the probe from binding to T-rich sequences that are found fairly commonly in eukaryotic DNA.

Whether or not to use a prehybridization solution containing formamide is largely a matter of personal preference. Both versions of these solutions give excellent results and neither has clear-cut advantages over the other. However, hybridization in 50% formamide at 42°C is less harsh on nitrocellulose filters than is hybridization at 68°C in aqueous solution. Offsetting this advantage is the two- to threefold slower rate of hybridization in solutions containing formamide.

To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength (6× SSC or 6× SSPE) at a temperature that is 20–25°C below T_m (see Chapter 9, pages 9.50–9.51). Both solutions work equally well when hybridization is carried out in aqueous solvents. However, when formamide is included in the hybridization buffer, 6× SSPE is preferred because of its greater buffering power.

Prehybridization solution

Either

50% formamide
6× SSC (or 6× SSPE)
0.05× BLOTTO

or

6× SSC (or 6× SSPE)
0.05× BLOTTO

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by stirring on a magnetic stirrer with Dowex XG8 mixed-bed resin for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

1× BLOTTO: Bovine Lacto Transfer Technique Optimizer (Johnson et al. 1984) is 5% nonfat dried milk dissolved in water containing 0.02% sodium azide. It should be stored at 4°C. 1× BLOTTO is as effective a blocking agent as Denhardt's reagent, but much less expensive. BLOTTO should not be used in combination with high concentrations of SDS, which will cause the milk proteins to precipitate. If background hybridization is a problem, NP-40 may be added to the hybridization solution to a final concentration of 1%. BLOTTO should not be used as a blocking agent when radiolabeled RNA is used as the hybridization probe, because of the possibility that the dried milk may contain significant amounts of RNAase activity.

Caution: Sodium azide is poisonous. It should be handled with great care wearing gloves, and solutions containing it should be clearly marked.

5. Denature ^{32}P-labeled double-stranded DNA probe by heating for 5 minutes to 100°C. Chill the probe rapidly in ice water. Single-stranded probe need not be denatured. Add the probe to the prehybridization solution covering the filters. Incubate at the appropriate temperature until $1-3 \times C_o t_{1/2}$ is achieved (see Chapter 9, page 9.48). During the hybridization, the containers holding the filters should be tightly closed to prevent the loss of fluid by evaporation.

   Alternatively, the probe may be denatured by adding 0.1 volume of 3 N NaOH. After 5 minutes at room temperature, transfer the probe to ice water and add 0.05 volume of 1 M Tris · Cl (pH 7.2) and 0.1 volume of 3 N HCl. Store the probe in ice water until it is needed.

   Between 2×10^5 and 1×10^6 cpm of ^{32}P-labeled probe (sp. act. $\geq 5 \times 10^7$ cpm/μg) should be used per milliliter of prehybridization solution. Using more probe will cause the background of nonspecific hybridization to increase; using less will reduce the rate of hybridization.

6. When the hybridization is completed, remove the hybridization solution and immediately immerse the filters in a large volume (300–500 ml) of 2× SSC and 0.1% SDS at room temperature. Agitate the filters gently, and turn them over at least once during washing. After 5 minutes, transfer the filters to a fresh batch of wash solution and continue to agitate them gently. Repeat the washing procedure twice more. At no stage during the washing procedure should the filters be allowed to dry.

   Hybridization mixtures containing radiolabeled single-stranded probes may be stored at 4°C for several days and reused without further treatment. However, hybridization mixtures containing complementary strands of DNA should be discarded since there is no satisfactory way to denature the double-stranded DNA that forms during the first round of hybridization.

7. Wash the filters twice for 1–1.5 hours in 300–500 ml of a solution of 1× SSC and 0.1% SDS at 68°C. At this point, the background is usually low enough to put the filters on film. If the background is still high or if the experiment demands washing at high stringencies, immerse the filters for 60 minutes in 300–500 ml of a solution of 0.2× SSC and 0.1% SDS at 68°C.

8. Dry the filters in the air at room temperature on paper towels. Arrange the filters (numbered side up) on a sheet of Saran Wrap. Apply adhesive dot labels marked with radioactive ink to several asymmetric locations on the Saran Wrap. These markers serve to align the autoradiograph with the filters. Cover the labels with Scotch Tape. This prevents contamination of the film holder or intensifying screen with the radioactive ink.

   Radioactive ink is made by mixing a small amount of ^{32}P with waterproof black drawing ink. We find it convenient to make the ink in three grades: very hot (>2000 cps on a hand-held minimonitor), hot (>500 cps on a hand-held minimonitor), and cool (>50 cps on a hand-held minimonitor). Use a fiber-tip pen to apply ink of the desired hotness to the adhesive labels. Attach radioactive-warning tape to the pen, and store it in an appropriate place.

9. Cover the filters with a second sheet of Saran Wrap. Expose the filters to X-ray film (Kodak XAR-2 or equivalent) for 12–16 hours at −70°C with an intensifying screen (see Appendix E).

10. Develop the film and align it with the filters using the marks left by the radioactive ink. Use a nonradioactive fiber-tip pen to mark the film with the positions of the asymmetrically located dots on the numbered filters. Tape a piece of clear Mylar or other firm transparent sheet to the film. Mark on the clear sheet the positions of positive hybridization signals. Also mark (in a different color) the positions of the asymmetrically located dots. Remove the clear sheet from the film. Identify the positive colonies by aligning the dots on the clear sheet with those on the agar plate.

    Some batches of nitrocellulose filters swell and distort during hybridization and subsequent drying, so that it becomes difficult to align the two sets of dots. This problem can be alleviated to some extent by autoclaving the dry filters between pieces of damp Whatman 3MM paper before use (10 lb/sq. in. for 10 minutes on liquid cycle). Nylon membranes do not suffer from this problem.

11. Using a sterile toothpick, transfer each positive bacterial colony into 1–2 ml of LB medium containing the appropriate antibiotic. Often, the alignment of the filters with the plate does not permit identification of an individual hybridizing colony. In this case, several adjacent colonies should be pooled. The culture is grown for several hours and then diluted and replated on agar plates so as to obtain approximately 500 colonies per plate. These colonies are then screened a second time by hybridization. A single, well-isolated, positive colony should be picked from the secondary screen and used for further analysis.

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